



Enhanced wound healing by topical administration of mesenchymal stem cells transfected with stromal cell-derived factor-1



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ABSTRACT

The objective of this study was to investigate the ability of mesenchymal stem cells (MSC) genetically engineered with stromal cell-derived factor-1 (SDF-1) to heal skin wounds. When transfected with SDF-1 plasmid DNA, MSC which were isolated from the bone marrow of rats, secreted SDF-1 for 7 days. In vitro cell migration assay revealed that the SDF-1-engineered MSC (SDF-MSC) enhanced the migration of MSC and dermal fibroblasts to a significantly greater extent than MSC. The SDF-MSC secreted vascular endothelial growth factor, hepatocyte growth factor, and interleukin 6 at a significantly high level. A skin defect model of rats was prepared and MSC and SDF-MSC were applied to the wound to evaluate wound healing in terms of wound size and histological examinations. The wound size decreased significantly faster with SDF-MSC treatment than with MSC and PBS treatments. The length of the neoepithelium and the number of blood vessels newly formed were significantly larger. A cell-tracing experiment with fluorescently labeled cells demonstrated that the percent survival of SDF-MSC in the tissue treated was significantly high compared with that of MSC. It was concluded that SDF-1 genetic engineering is a promising way to promote the wound healing activity of MSC for a skin defect.

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1. Introduction

Wound healing is a complex biological process comprising three phases: inflammation, tissue formation including angiogenesis and granulation, and tissue remodeling [1]. Both acute and chronic wounds continue to be a major clinical problem. Non-healing wounds may result from a variety of causes, including large defects, diabetes, ischemia, and complications from radiation therapy. Multiple impairments in cellular responses, including the production of growth factors and cytokines, the recruitment of cells into injured tissues, contribute to non-healing wounds [1,2]. Various strategies, including growth factor [2–5], gene [6–8], and stem cell therapies [9–11], have been used to enhance the healing of non-healing wounds. Stem cell-based therapy is an attractive approach for the treatment of wounds with multiple impairments.

Mesenchymal stem cells (MSC), which are referred to as multipotent stromal progenitor cells, have been shown to promote tissue repair in numerous studies [9–13]. Transplantation of MSC

can improve wound healing through cell differentiation and the release of paracrine factors [9–11,14]. However, the poor viability of MSC at the transplanted site often decreases their therapeutic potential [15,16]. It is important to improve the survival of transplanted MSC and enhance the secretion of factors and their biological functions in vivo.

Genetic engineering of MSC is a potential method to break through these problems. Much research has reported the therapeutic effects of MSC genetically engineered using viral vectors [8,17,18], but the strategy using viral vectors is not available for clinical cell therapy. It is necessary for clinical cell therapy to use non-viral vectors of gene transfection.

We have developed a spermine-pullulan of a non-viral vector for the gene transfection of MSC [19,20]. The spermine-pullulan is a cationic carrier of spermine-introduced pullulan which can interact with negatively charged plasmid DNA, and can be internalized into MSC by way of a sugar-specific-asialo-protein receptor that is expressed on the surface of MSC [21]. As a result, the complex of spermine-pullulan and plasmid DNA has been demonstrated to be feasible in gene transfection with low cytotoxicity and high specificity for MSC [19–21]. We have previously reported that MSC genetically engineered with spermin-pullulan showed better survival of cells and their therapeutic potential in vivo [21,22].

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Stromal cell-derived factor-1 (SDF-1, CXCL12) is known as a chemokine that plays a central role in normal wound healing [7]. SDF-1 functions in inflammation reactions, leukocyte development, and the mobilization and recruitment of stem and progenitor cells including hematopoietic stem cells (HSC) and MSC, and other CXCR4 (SDF-1 receptor)-expressing cells [23,24]. Consequently, SDF-1 contributes to cell-based vascularization and skin regeneration [25]. Moreover, SDF-1 increased the survival and growth of CXCR4-expressing stem cells, such as MSC, both in vitro [26] and in vivo [18,27]. Some researchers have reported that the local administration of SDF-1 enhanced wound healing [4,7,17,23].

In this study, we evaluated the therapeutic effect of local administration of MSC genetically engineered with SDF-1 on a full-thickness skin defect model of rats. The MSC were transfected with the complex of spermine-pullulan and SDF-1 plasmid DNA. When treated with SDF-1-engineered MSC, wound healing of skin defects was assessed in terms of size and histological examinations. In addition, we examined the mechanism of the therapeutic effect with MSC genetically engineered in terms of cell migration and growth factor secretion.

2. Materials and methods

2.1. Materials

Pullulan with an average molecular weight of 47,300 was purchased from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). Spermine was purchased from Sigma Chemical Co. (St. Louis, MO). N,N'-carbonyldiimidazole (CDI) and dehydrated dimethyl sulfoxide were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and used as obtained.

2.2. MSC isolation and culture

MSC culture was performed according to the method previously described [21]. Briefly, 3-wk-old Fisher 344 rats (Japan SLC, Inc., Shizuoka, Japan) were sacrificed, and the bone marrow was harvested by flushing femurs and tibiae with alpha-minimum essential medium (α -MEM; Invitrogen Corporation, Ltd., Carlsbad, CA) supplemented with 15 vol% bovine fetal calf serum (FCS) and 1 wt% penicillin–streptomycin (control medium). The bone marrow cell mixture was placed in a 10 cm tissue culture dish (430167; Corning Inc., Cambridge, MA) and cultured in the control medium at 37 °C in 5% CO₂ and 95% air atmospheric condition. The medium was refreshed on the third day after isolation to remove non-adherent cells and continuously refreshed every 3 days. Cells of 2–3 passages in a 70–80% confluent condition were used for the following experiments.

2.3. Dermal fibroblast isolation and culture

Dermal fibroblasts were obtained from 3-wk-old Fisher 344 rats as previously described [28]. Briefly, the skin specimens were cut into small pieces with surgical scissors and placed in a 10 cm tissue culture dish. The skin specimens were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen Corp.) supplemented with 10 vol% FCS and 1 wt% penicillin–streptomycin at 37 °C in 5% CO₂ and 95% air atmospheric condition, and the medium were changed every 3 or 4 days. Outgrowing fibroblasts were dissociated with 0.25 wt% trypsin-EDTA (Invitrogen Corp.) and passaged. Passages 3–5 were used for the following experiments.

2.4. Preparation of plasmid DNA

The plasmid DNA used was a plasmid coding human SDF-1 alpha (pORF-hSDF-1 α ; InvivoGen, San Diego, CA). The plasmid DNA was propagated in *Escherichia coli* (strain DH5 α) and purified by the QIAGEN plasmid Mega kit (Qiagen K.K., Tokyo, Japan) according to the manufacturers' instructions. Both the yield and purity of plasmid DNA were evaluated by UV spectroscopy (DU800 spectrometer; Beckman Coulter, Germany). The absorbance ratio at wavelengths of 260–280 nm for plasmid DNA solution was measured to be between 1.8 and 2.0.

2.5. Preparation of cationized pullulan

Spermine was chemically introduced into the hydroxyl groups of pullulan by a CDI activation method [20]. Briefly, 9.28×10^{-3} mol of spermine and 1.39×10^{-3} mol of CDI were added to 50 ml dehydrated dimethyl sulfoxide containing 50 mg pullulan. Following agitation at room temperature for 20 h, the reaction mixture was dialyzed against double-distilled water (DDW) for 2 days. Then, the solution dialyzed was freeze-dried to obtain samples of spermine-introduced pullulan

(spermine-pullulan). When determined by conventional elemental analysis, the molar percentage of spermine introduced into the hydroxyl groups of polysaccharides was 11.0 mol%.

2.6. Preparation of spermine-pullulan–plasmid DNA complexes

Polyion complexes (PIC) were prepared by mixing spermine-pullulan and the SDF-1 plasmid in aqueous solution. Briefly, spermine-pullulan was dissolved in DDW and mixed with an equal volume of PBS containing 100 μ g plasmid DNA (100 μ g/ml), and then left for 15 min at room temperature. The PIC composition was calculated on the basis of the nitrogen number of spermine-pullulan (N) per the phosphorus number of plasmid DNA (P) (N/P ratio). In this study, the PIC were prepared at the N/P ratio of 3.0 [22].

2.7. Gene transfection of MSC and the time course of SDF-1 secretion

MSC (1×10^4 cells/cm²) were cultured with spermine-pullulan–plasmid DNA complexes at the plasmid DNA dose of 0.5 mg/cm² at 37 °C for 4 h in OPTI MEM (Invitrogen Corp.) to allow cells to be genetically engineered. Then, the medium was changed to the control medium, and MSC genetically engineered with SDF-1 plasmid DNA (SDF-MSC) were incubated further for 24 h. The level of human SDF-1 expressed in the culture medium ($n = 3$) was measured by ELISA (Human CXCL12/SDF-1 α ELISA Quantikine Kit; R&D Systems Inc., Minneapolis, MN). The total protein of each well was determined with BCA Protein Assay Reagent (Thermo Fisher Scientific, Inc., Waltham, MA) to normalize the influence of the number variance of cells.

To investigate the time course of SDF-1 secreted from MSC and SDF-MSC, the cells were cultured for 7 days in control medium, which was collected each day and replaced. The amount of SDF-1 in the medium was determined by the ELISA ($n = 3$).

2.8. Transmembrane migration assay

To evaluate the effect of MSC and SDF-MSC on MSC and dermal fibroblasts migration, a transmembrane migration assay was performed using 24-well Transwell inserts (#3422; Corning Corp.). MSC were seeded in the bottom chamber at a density of 1×10^4 cells/cm² and cultured for 24 h in the control medium. MSC were divided into two groups with MSC and SDF-MSC. In the SDF-MSC group, MSC were transfected as described above and the medium (control medium or DMEM with 10 vol% FBS and 1 wt% penicillin–streptomycin) was replaced 24 h after transfection. In the MSC group, the medium (control medium or DMEM with 10 vol% FBS and 1 wt% penicillin–streptomycin) was replaced similarly. Then, 6.5 mm-diameter Transwell chambers (upper chamber) with polycarbonate membrane inserts (8 μ m pore size) were placed in each group and well. MSC in the control medium or dermal fibroblasts in DMEM with 10 vol% FBS and 1 wt% penicillin–streptomycin were seeded into the upper compartment of the upper chamber (1×10^4 cells/well), followed by incubation at 37 °C for 24 h. Then, the number of cells that had migrated through the membrane to the lower compartment of the upper chamber was counted after their trypsinization ($n = 6$). The control bottom chambers contain medium (control medium or DMEM with 10 vol% FBS and 1 wt% penicillin–streptomycin) without cells.

2.9. Secretion of growth factors and cytokines from MSC and SDF-MSC

To evaluate the secretion of growth factors and cytokines, the conditioned medium of MSC and SDF-MSC was analyzed with commercial ELISA kits. MSC and SDF-MSC were cultured in a 12-well plate for 72 h and the conditioned medium was collected ($n = 4$). The growth factor and cytokine levels in the conditioned medium were measured by ELISA (Rat VEGF, Rat HGF, Rat IL-6, Rat TGF- β , Human FGF2, Rat PDGF-bb ELISA Quantikine Kit; R&D Systems Inc.). The total protein in each well was determined with BCA Protein Assay Reagent (Thermo Fisher Scientific, Inc.) to normalize the influence of the number variance of cells.

2.10. Wound healing model and cells transplantation

Eight-wk-old Fisher 344 rats ($n = 18$, 6 rats in each group) were purchased from Japan SLC (Japan SLC, Inc.) and each rat was anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital (Abbot Laboratories, North Chicago, IL). After shaving and depilating the rats, two 15-mm full-thickness skin defects including the panniculus carnosus were created on each side of the dorsal midline. The rats were divided into three groups and MSC or SDF-MSC (1.0×10^6 cells) in 500 μ l PBS or 500 μ l PBS alone were homogeneously administered into the subcutaneous tissue around the wound defect. The wound was covered with polyurethane film (Tegaderm; 3M HealthCare, Borken, Germany).

2.11. Wound analysis

The skin wounds were photographed on different days after treatment with MSC and SDF-MSC or PBS. The wound area was analyzed by tracing the wound

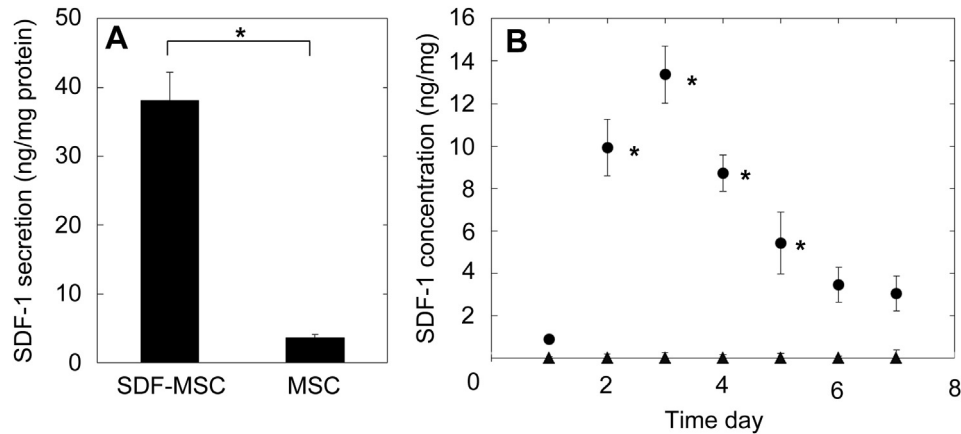


Fig. 1. (A) Secretion of SDF-1 from SDF-MSC and MSC for 24 h. * $p < 0.05$; significant difference between the two groups. (B) Time course of SDF-1 secreted from SDF-MSC (●) and MSC (▲). * $p < 0.05$; significant difference in the concentration of MSC at the corresponding time.

margin and calculating the area using ImageJ software (version 1.38). The percentage of wound closure was calculated as follows: [(Area of original wound – Area of actual wound)/Area of original wound] $\times 100$.

2.12. Histological and immunohistochemical examinations

The rats were sacrificed by CO_2/O_2 inhalation 7 days after treatment, and then the wounds were harvested with the surrounding tissue. The tissue specimens were fixed with 4 wt% paraformaldehyde in PBS at 4 °C for 24 h and embedded in paraffin to prepare histological sections. The 4 μm -thick sections were stained with hematoxylin and eosin. Using a light microscope, the neoeepithelium length of each specimen was measured from the marginal skin to the end of the neoeepithelium on each side of cross-sections.

Immunohistochemical staining with von Willebrand factor was performed to identify blood vessels in the wound granulation tissue. The 4 μm -thick paraffin-embedded sections were deparaffinized. After pretreatment with 0.1 wt% trypsin (Vector Laboratories Inc., Burlingame, CA) for 15 min at 37 °C, 0.3% hydrogen peroxide for 15 min at room temperature, and Blocking I (Nacalai Tesque Inc., Kyoto, Japan) for 10 min at room temperature, anti-Von Willebrand factor rabbit polyclonal antibody (1:2500 dilution, #A0082; Dako North America, Inc., Carpinteria, CA) was applied as the primary antibody overnight at 4 °C. Histofine Simple Stain Rat MAX PO (MULTI) (#414191; Nichirei Biosciences Inc., Tokyo, Japan) was applied as the secondary antibody. The color was developed by DAB (3,3'-diaminobenzidine tetrahydrochloride) for 1 min at room temperature. Hematoxylin was used for counterstaining. Ten different fields within the wound granulation tissue were randomly selected from each section, and the number of blood vessels

per field was counted. The average of 10 values was used to determine the blood vessel density.

2.13. Viability analysis of cells transplanted

Wounds were created on the dorsum of rats as described above and MSC or SDF-MSC (1×10^6 cells) in PBS (500 μl) was subcutaneously administered around the wound. Before that, MSC or SDF-MSC was fluorescently labeled with PKH26 (Sigma–Aldrich Co., St. Louis, MO) according to the manufacturer's protocol. The rats were sacrificed 7 and 14 days after treatment. The wounds with their surrounding tissue were harvested, the epidermis removed, minced into small pieces, and digested with 2 mg/ml collagenase D (Roche Diagnostics GmbH, Mannheim, Germany) in Hanks' Balanced Salt Solution (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan). The remaining tissues were separated from cells using a cell strainer (70 μm , BD Falcon; BD Biosciences Inc., Billerica, MA) and the cells were collected by centrifugation at 2000 rpm and 4 °C for 5 min. Then, the cells were dispersed in 1 ml PBS and the cell nuclei were stained with Hoechst 33258 (B-1155; Sigma–Aldrich Co.). PKH26-positive cells were counted with a cell counter (BMS-OCC01; Bio Medical Science, Tokyo, Japan) using a fluorescence microscope (AX80 Provis; Olympus Ltd., Osaka, Japan). The survival percentage of transplanted cells was expressed: [number of PKH26-positive cells/number of transplanted cells (1×10^6)] $\times 100(\%)$.

Wound tissues were harvested 7 and 14 days after treatment, fixed with 4 wt% paraformaldehyde in PBS at 4 °C for 24 h, embedded in O.C.T. compound (Sakura Finetek Japan, Tokyo, Japan), and sectioned (6 μm thickness) randomly. The sections were stained with Hoechst 33258 for 15 min at room temperature and mounted

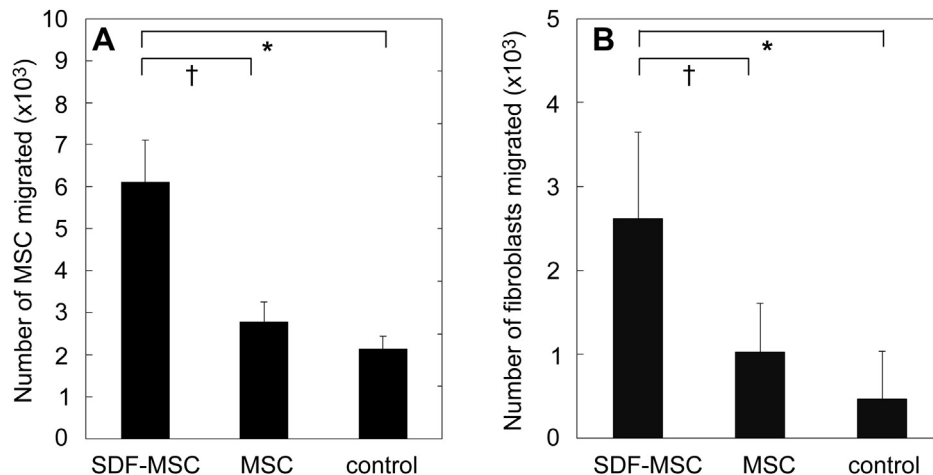


Fig. 2. Number of MSC (A) and fibroblasts migrated (B) 24 h after co-culture with SDF-MSC and MSC or without cells (control). * $p < 0.05$; significant difference from the number of control. † $p < 0.05$; significant difference in the number of MSC.

with a fluorescence mounting medium (S3032, Dako North America, Inc.). The fluorescence images were taken to view the histology.

2.14. Statistical analysis

Data are expressed as the means \pm standard deviations. Data were statistically analyzed with the Tukey–Kramer paired comparison test while significance was accepted at $p < 0.05$.

3. Results

3.1. SDF-1 secretion from SDF-MSC

Fig. 1(A) shows the concentration of human SDF-1 secreted from SDF-MSC and MSC. SDF-MSC secreted a significantly greater amount of SDF-1 than MSC.

Fig. 1(B) shows the time course of human SDF-1 secreted by SDF-MSC. The level of SDF-1 secretion increased with the time up to 3 days after gene transfection, and then decreased over 7 days.

3.2. Biological functions of SDF-MSC

Fig. 2(A) and (B) shows the number of MSC and dermal fibroblasts that had migrated 24 h after co-culture with SDF-MSC and MSC. Larger amounts of MSC and dermal fibroblasts were migrated by co-culture with SDF-MSC than with MSC.

Fig. 3 shows the levels of growth factors and cytokines from SDF-MSC and MSC. SDF-MSC secreted a significantly larger amount of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and interleukin (IL)-6 than MSC. On the other hand, no significant differences in the secretion of transforming growth factor- β 1 (TGF- β 1), basic fibroblast growth factor (bFGF), or platelet-derived growth factor (PDGF)-BB were observed between SDF-MSC and MSC (data not shown for bFGF and PDGF-BB).

3.3. In vivo wound healing effect of SDF-MSC

Fig. 4 shows the gross appearance of wounds after treatment with SDF-MSC, MSC and PBS. The skin wounds had almost closed 14 days after treatment with cells, irrespective of SDF-1 engineering. However, in the PBS-treated control group, wound healing was delayed.

Fig. 5 shows the time course of the wound area after treatment with SDF-MSC, MSC and PBS. In the groups treated with both cells, the wound areas were significantly smaller than the control group 7, 10, and 14 days after treatment. Moreover, after 5, 10, and 14 days, the areas of wounds treated with SDF-MSC were significantly smaller than those treated with MSC.

Fig. 6 shows histological results of wounds 7 days after treatment. The neoepithelium length in wounds was significantly longer with SDF-MSC treatment than in the PBS-treated, control group.

Fig. 7 shows the immunohistochemical results. The density of blood vessels newly formed in tissue granulation treated with SDF-MSC was significantly higher than in MSC and control groups.

3.4. Viability of transplanted cells

Fig. 8(A) shows the percent survival of PKH26-positive transplanted cells 7 and 14 days after treatment with SDF-MSC and MSC. The survival percentage was significantly higher in the SDF-MSC group than in the MSC group.

Fig. 8(B) shows a histological image 14 days after treatment with SDF-MSC. PKH26-positive transplanted cells were detected in the subdermal area in the margin of the wound where cells were administered. The area of PKH26-positive cells detected after 14 days was larger in the tissue with SDF-MSC treatment than with MSC treatment.

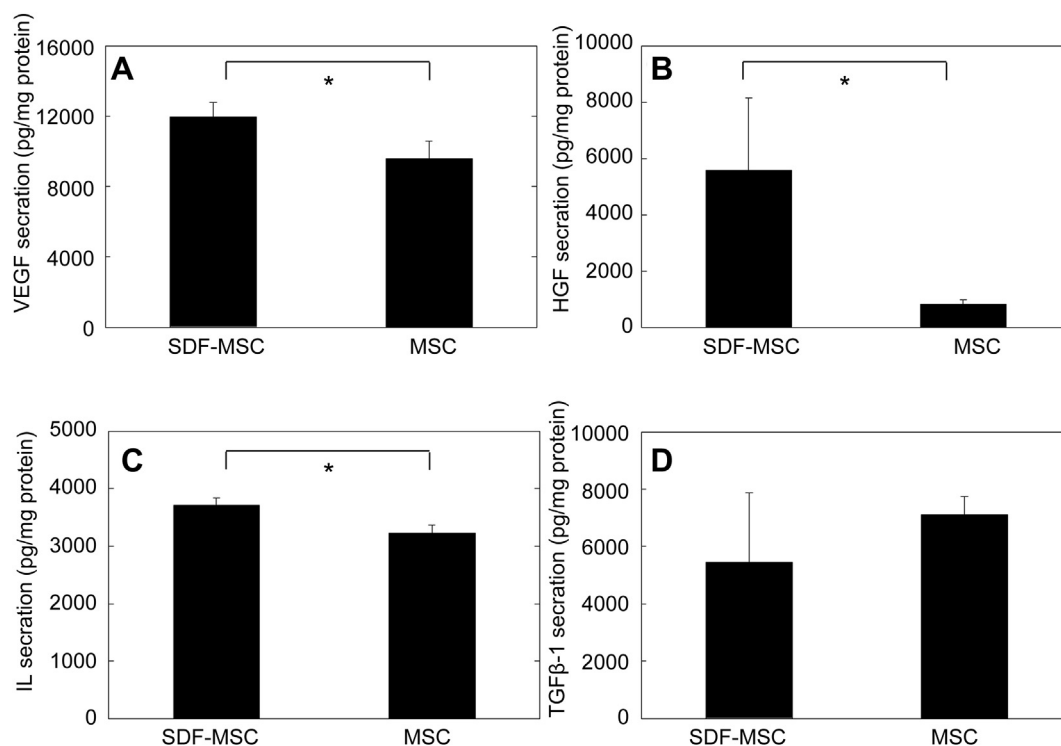


Fig. 3. Secretion levels of VEGF (A), HGF (B), IL-6 (C), and TGF- β 1 (D) 72 h after SDF-MSC and MSC culture. * $p < 0.05$; significant difference in the level of MSC.

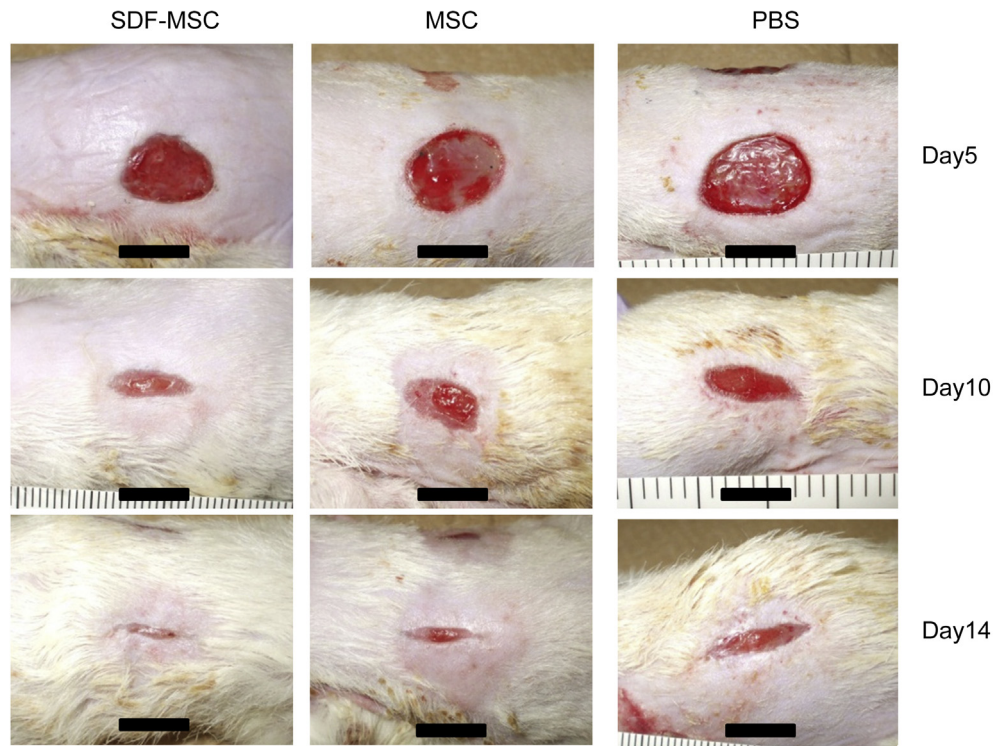


Fig. 4. Gross appearance of skin wounds at 5, 10, and 14 days after treatment with SDF-MSC and MSC or PBS. Scale bar = 10 mm.

4. Discussion

Wound therapy remains a clinical challenge and much effort has been focused on the development of novel therapeutic approaches for wound treatment. Growth factor therapy [2–4], gene therapy with genes encoding for growth factors or cytokines [6–8], and stem cell therapy [9–11] have been shown to improve wound healing. In order to accelerate wound healing, a combination of these therapies has been reported. Recently, stem cell-based therapy, particularly the combination of gene and stem cell therapies, represents the most attractive therapy for wound healing [6].

Among the many factors contributing to non-healing wounds, the impaired production of cytokines and reduced angiogenesis

and tissue regeneration are crucial. Since MSC secreted several cytokines and ECM molecules, they are used as a source of soluble signals that regulate cellular responses in wound healing [10,14]. Several studies have reported that the administration of MSC genetically engineered with genes encoding growth factors or cytokines accelerates wound healing [8,17]. In most studies, viral vectors are used for gene transfection because of high transfection efficiency. However, considering the clinical application, it is preferable to use non-viral vectors of gene transfection. This study experimentally demonstrated that spermine-pullulan of a non-viral vector was effective in enhancing the gene expression of MSC and promoting wound healing.

MSC genetically engineered with the complex of spermine-pullulan and SDF-1 plasmid DNA (SDF-MSC) secreted SDF-1 at higher amounts than MSC over 7 days (Fig. 1). It is possible that this release profile of SDF-1 is suitable for the acceleration of wound healing. This is because it is important for wound healing that the dynamics of SDF-1 is controlled temporally in the early stage [24,29]. SDF-1 is constitutively expressed in normal skin and may contribute to tissue homeostasis. When homeostasis is disrupted by injury, SDF-1 is upregulated for 4–7 days at the wound margins [24,30]. SDF-1 has a central role in the early stage of wound healing [7]. On the other hand, it is reported that SDF-1 is severely downregulated in a diabetic and irradiated wound, and this SDF-1 downregulation contributes to the impairment of wound healing [30–32]. The administration of SDF-1 promotes the healing of acute and chronic skin wounds [4,7,17].

The in vitro transmembrane migration assay shows that MSC and dermal fibroblasts migrated significantly strongly in the co-culture with SDF-MSC than with MSC (Fig. 2). It has been reported that MSC expressed CXCR4 receptors and migrated by SDF-1 through the SDF-1-CXCR4 signaling pathway [18,33]. This result indicates that SDF-1 secreted from SDF-MSC had a biological function to enhance the migration of MSC and dermal fibroblasts.

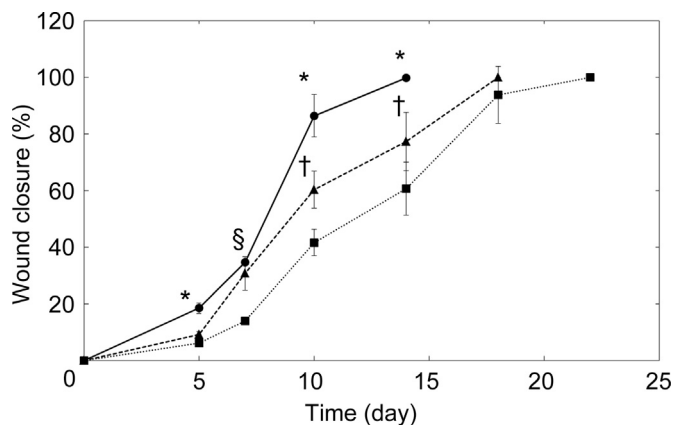


Fig. 5. Time courses of skin wound closure after treatment with SDF-MSC (●) and MSC (▲) or PBS (■). * $p < 0.05$; significant difference in the percent closure of MSC and PBS. † $p < 0.05$; significant difference in the percent closure of PBS. § $p < 0.05$; significant difference in the percent closure of PBS.

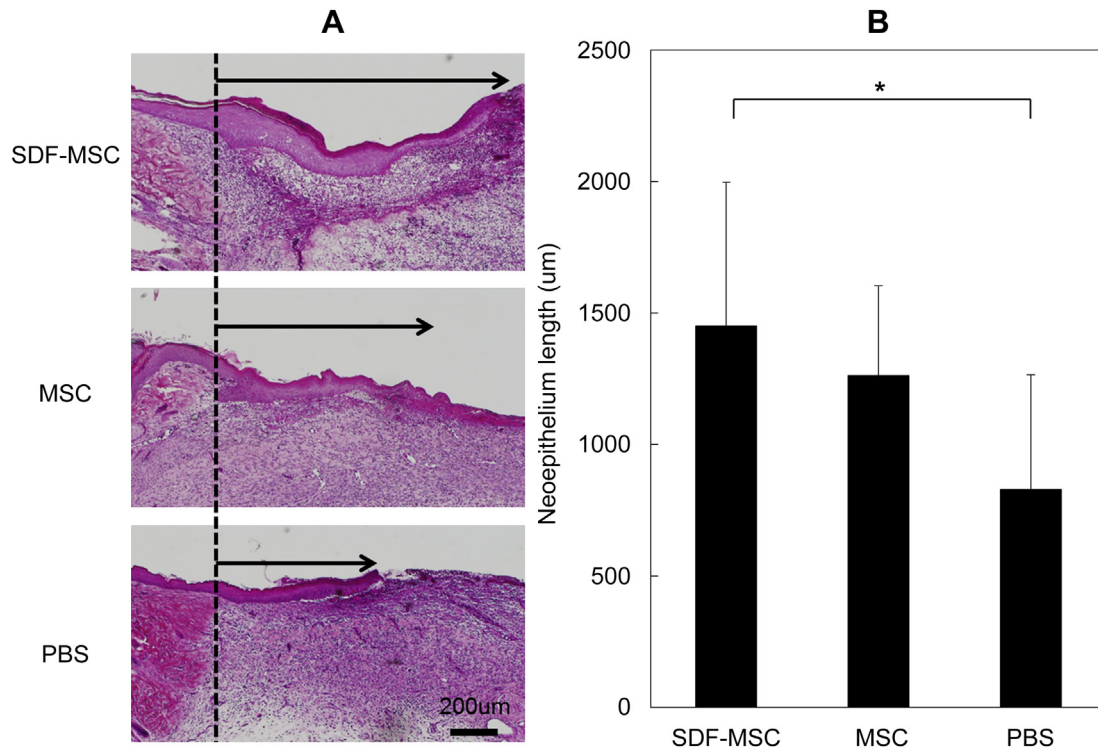


Fig. 6. (A) Histological images of skin wounds 7 days after treatment with SDF-MSC and MSC or PBS. Black arrow indicates neopithelium. Scale bar = 200 μm. (B) Neopithelium length of skin wounds 7 days after treatment with SDF-MSC and MSC or PBS. * $p < 0.05$; significant difference in the length of PBS.

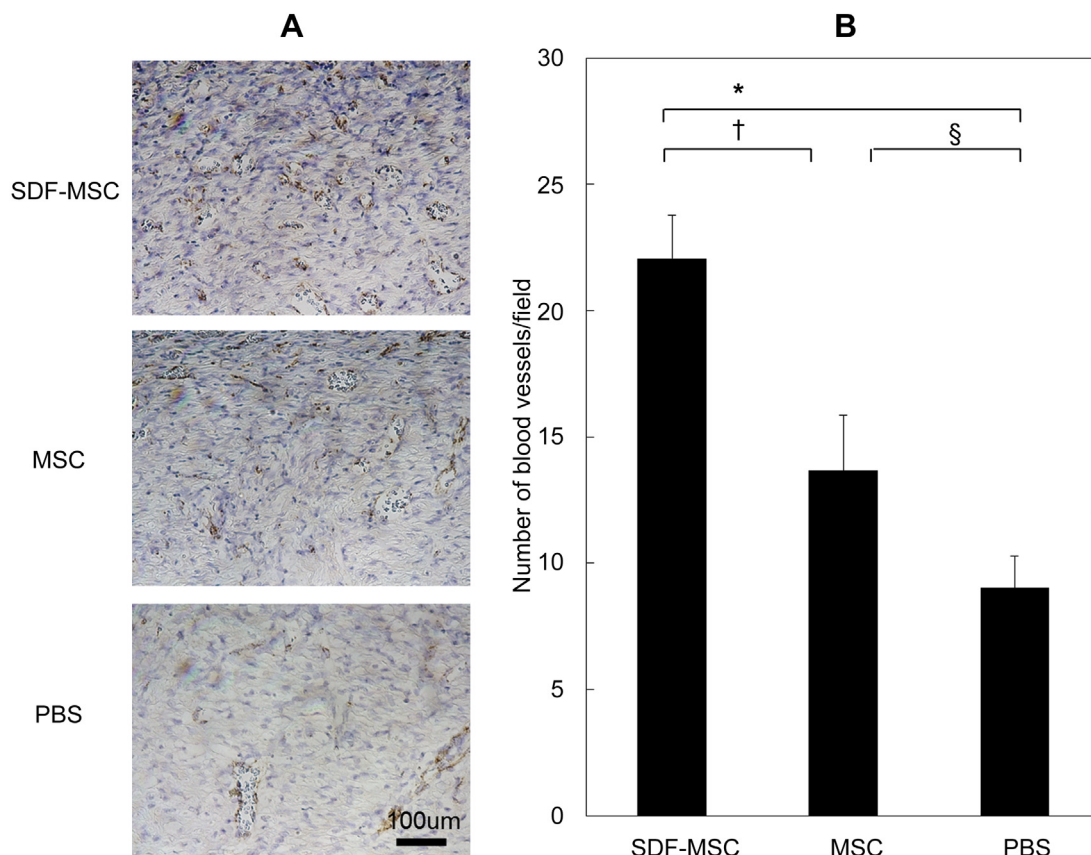


Fig. 7. (A) Immunohistochemical staining images of skin wounds 7 days after treatment with SDF-MSC and MSC or PBS. (B) Vascular density within skin wounds 7 days after treatment with SDF-MSC and MSC or PBS. * $p < 0.05$; significant difference between SDF-MSC and PBS. † $p < 0.05$; significant difference between SDF-MSC and MSC. § $p < 0.05$; significant difference between MSC and PBS. Scale bar = 100 μm.

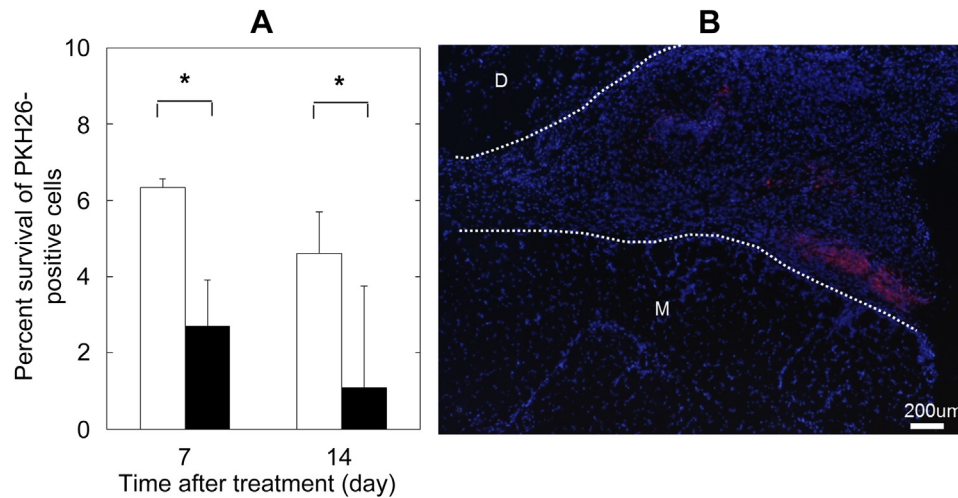


Fig. 8. (A) Percent survival of PKH26-positive cells 7 and 14 days after treatment with SDF-MSC (open bar), MSC (closed bar). * $p < 0.05$; significant difference in the percent survival of MSC. (B) Histological image of skin wound treated with PKH26-positive SDF-MSC 14 days after treatment. PKH26-positive cells: red, cell nuclei: blue, D = dermis, M = muscle. Scale bar = 200 μm (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

However, dermal fibroblasts do not express CXCR4 receptors [34]. It has been demonstrated that paracrine factors released by SDF-MSC act on dermal fibroblasts and promote their proliferation and migration [35–38]. bFGF, TGF- β 1, PDGF-bb, and IL-6 are known as cytokines to enhance dermal fibroblast migration [2,35,39]. IL-6 was secreted in a significantly large amount by SDF-MSC compared with MSC (Fig. 3). It is highly conceivable that IL-6 secreted from SDF-MSC induced the acceleration of dermal fibroblast migration. Dermal fibroblast responses are essential for wound healing of skin [1,40]. The initial responses of dermal fibroblasts include proliferation and migration into the wound. IL-6 is an essential factor in dermal fibroblast migration [39] and also stimulates keratinocyte migration [41]. In addition, SDF-MSC secreted a greater amount of HGF than MSC (Fig. 3). It is reported that HGF is one of the growth factors to enhance wound healing [3]. HGF stimulates the proliferation and migration of keratinocytes [42], and increases neovascularization and the formation of granulation tissue [3]. Taken together, SDF-MSC may promote skin wound healing by accelerating the activity of skin cells, such as keratinocytes and dermal fibroblasts.

The *in vivo* study shows that treatment with SDF-MSC promoted the wound healing of full-thickness skin defects. In gross appearance, wounds treated with MSC or SDF-MSC healed more rapidly than those treated with PBS. Moreover, better wound healing was observed with SDF-MSC treatment than with MSC (Figs. 4 and 5). In the wound-healing process, angiogenesis and tissue regeneration are particularly important. It is necessary to promote these processes that the recruitment of cells from the surrounding tissue and bone marrow to the wound site, such as dermal fibroblasts, keratinocytes, local progenitor cells, stem cells including HSC and MSC [9]. The promotion of wound healing can be explained in terms of cell recruitment. SDF-1 plays a critical role in the mobilization and recruitment of stem cells, including HSC and MSC and other CXCR4-expressing cells, including keratinocytes, to the injured tissue [24,43–46]. The recruited cells would secrete several growth factors and cytokines, which are necessary to promote tissue regeneration [2,10,14]. It is possible that SDF-MSC enhance the recruitment of cells into wounds, resulting in promoted cellularity for wound healing.

Histological analysis demonstrated that the neopithelium length was longer in wounds treated with SDF-MSC than that with PBS (Fig. 6). The IL-6, HGF, and SDF-1 secreted from SDF-MSC would

enhance the activity of dermal fibroblasts and keratinocytes to promote re-epithelialization.

In immunohistochemical analysis, a higher density of blood vessels in wounds was observed in the treatment with SDF-MSC (Fig. 7). It is reported that SDF-1 enhances the mobilization and recruitment of HSC, and consequently contributes to cell-based angiogenesis and vascularization [24,25,47]. Gallagher et al. [30] reported that the local administration of SDF-1 into skin defect wounds increased the recruitment of HSC, resulting in enhanced wound healing. Our previous study [23] and Salcedo et al. [48] reported that the local application of SDF-1 to the skin induced angiogenesis and neovascularization. In this study, SDF-1 secreted from SDF-MSC may increase the recruitment of HSC and enhanced angiogenesis. In addition, SDF-MSC secreted a larger amount of VEGF and HGF than MSC (Fig. 3). VEGF and HGF also have functions to enhance angiogenesis and vascularization [3,49]. It is possible that the combination of these angiogenic factors promotes neovascularization. We can say with certainty that angiogenesis by the factors secreted from SDF-MSC accelerated the transportation of necessary cells and humoral factors, and enhanced granulation tissue formation, resulting in promoted wound healing.

The survival percentage of SDF-MSC was higher than that of MSC (Fig. 8). It is well known that SDF-1 increases the survival and growth of MSC both *in vitro* [26] and *in vivo* [18,27]. Based on these findings, it is likely that SDF-1 secreted from SDF-MSC increased their *in vivo* survival. Genetic engineering with SDF-1 enabled enhanced survival of MSC and the secretion of factors for cell migration and angiogenesis *in vivo*. As the result, enhanced wound healing was observed by treatment with SDF-MSC.

5. Conclusions

MSC were genetically engineered with a complex of spermine-pullulan and SDF-1 plasmid DNA. Engineered MSC secreted biologically active SDF-1, which enhances the migration of MSC and dermal fibroblasts, and secreted VEGF, HGF, and IL-6 *in vitro*. When treating rat skin wounds, a larger number of engineered MSC were retained at the site and wound healing was promoted compared with non-engineered MSC. It is concluded that genetically engineered MSC functioned in a safe and effective therapeutic manner to promote wound healing.

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